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Targeting Class I_A PI3K Isoforms Selectively Impairs Cell Growth, Survival, and Migration in Glioblastoma

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Abstract

The phosphoinositide 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) pathway is frequently activated in human cancer and plays a crucial role in glioblastoma biology. We were interested in gaining further insight into the potential of targeting PI3K isoforms as a novel anti-tumor approach in glioblastoma. Consistent expression of the PI3K catalytic isoform PI3K p110 α was detected in a panel of glioblastoma patient samples. In contrast, PI3K p110 β expression was only rarely detected in glioblastoma patient samples. The expression of a module comprising the epidermal growth factor receptor (EGFR)/PI3K p110 α /phosphorylated ribosomal S6 protein (p-S6) was correlated with shorter patient survival. Inhibition of PI3K p110 α activity impaired the anchorage-dependent growth of glioblastoma cells and induced tumor regression *in vivo*. Inhibition of PI3K p110 α or PI3K p110 β also led to impaired anchorage-independent growth, a decreased migratory capacity of glioblastoma cells, and reduced the activation of the Akt/mTOR pathway. These effects were selective, because targeting of PI3K p110 δ did not result in a comparable impairment of glioblastoma tumorigenic properties. Together, our data reveal that drugs targeting PI3K p110 α can reduce growth in a subset of glioblastoma tumors characterized by the expression of EGFR/PI3K p110 α /p-S6.

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Introduction

Gliomas are the most common tumors of the central nervous system, accounting for 80% of malignant brain tumors. Among these, glioblastoma (GBM) represents the largest and most malignant subgroup. GBMs are generally characterized by a highly heterogeneous and highly infiltrative phenotype. Standard treatment of care includes surgical resection, followed by radiation and/or chemotherapy [1]. Despite this aggressive treatment, almost all GBM eventually recur and the median survival of GBM patients is only around one year [2,3]. Novel therapies are thus urgently needed.

Genetic alterations in GBM predominantly include amplifications or mutations in the gene encoding the epidermal growth factor receptor (*EGFR*). A rather characteristic mutation thereby leads to the truncated version EGFRvIII, present in about 20% of GBM [4], which possesses an in-frame deletion of exons 2 to 7 in the extracellular domain and whose expression has been shown to correlate with elevated downstream signaling activation [5,6]. Signaling via EGFR has also been demonstrated to contribute to resistance of GBM to radiation and chemotherapy [7]. Further

growth factors have been described to play important roles in GBM tumorigenesis. For instance, insulin-like growth factor I (IGF-I) seems to be important in GBM chemoresistance, whereas platelet-derived growth factor (PDGF) is thought to signal in an autocrine fashion in GBM cells, thereby contributing to proliferation and survival of GBM tumor cells [8,9]. Due to the fact that several different growth factors and their receptors play an important role in GBM biology, the treatment of one receptor alone might not lead to satisfying results. On the contrary, targeting a signaling molecule downstream of these growth factors might represent a successful treatment strategy.

The family of phosphoinositide 3-kinases (PI3Ks) lies downstream of several growth factor receptors. PI3Ks play an essential role in signal transmission and they are crucial in controlling cell proliferation, survival, and motility [10]. These enzymes phosphorylate membrane phosphoinositides on the inner side of the cell membrane on the D3 position of the inositol ring. These 3-phosphorylated membrane phosphoinositides then act as second messengers which are important for the activation of the downstream signaling pathway, including phosphorylation and thereby activation of Akt [11]. Further accounting to the

importance of PI3K signaling in cancer is the fact that the tumor suppressor gene phosphatase and tensin homologue deleted on chromosome 10 (*PTEN*) is frequently mutated in human cancers, including GBM (approximately 20–30%) [10,12–15]. *PTEN* acts as an antagonist of PI3K by dephosphorylating phosphatidylinositol-3,4,5-trisphosphate and thus reducing downstream signaling activity [16,17]. Additionally, *PIK3CA*, the gene encoding class I α PI3K isoform p110 α , has been shown to be mutated in GBM tumor samples. *PIK3CA* mutations were found in 0–27% of GBM tumor samples, depending on the study and the detection method used [14,15,18–21]. Furthermore, and also depending on the study and the method used, copy number increases of both *PIK3CA* and *PIK3CD* (encoding PI3K p110 δ) have been reported (ranging from approximately 0% to 60%) [14,19,21–23]. Taken together, deregulation of the PI3K/Akt/mTOR pathway is frequently detected in GBM (in around 88%) [12] and has been found to contribute to a variety of cellular responses, thus rendering this pathway and especially the PI3Ks interesting candidates for targeted therapies.

In the present study, we have investigated the expression of class I α PI3K isoforms in GBM tumor samples, cell lines, and *ex vivo* cultures. We have further investigated the involvement of the different isoforms in cell proliferation, survival, and migration. Our results show that individual class I α PI3K isoforms have selected cellular functions in GBM cells. The PI3K p110 α had a broad expression pattern in primary tumors and was associated with the phosphorylation status of the ribosomal S6 protein, which correlated with decreased patient survival. The PI3K isoform p110 α also appears to be essential for cell growth under anchorage-independent conditions and *in vivo*. Targeting PI3K p110 β impaired anchorage-independent growth and cell migration in GBM cells, while targeting PI3K p110 δ was ineffective. Thus the PI3K p110 α and p110 β isoforms appear to differentially contribute to critical cell responses in GBM, which is important when considering the future use of isoform-specific PI3K isoforms in this devastating malignancy.

Materials and Methods

Glioma Tissue Microarray

The study cohort comprised of low and high grade glioma samples from 103 patients operated at the Department of Neurosurgery, University Hospital Zurich (Zurich, Switzerland). Written informed consent was obtained from all the patients before study entry. The procedures were conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the Canton Zurich. Paraffin blocks of these tumors were reviewed by a neuropathologist and classified according to the World Health Organization (WHO) of brain tumors [24]. Representative tumor areas were marked on hematoxylin/eosin-stained slides and two cores (0.6 mm diameter) were punched from the donor block and transferred into the tissue microarray (TMA) recipient block, as recently described [25]. Additionally, the TMA contained four normal brain samples (in duplicates) and four cell lines (MCF-7, LN18, HT29, LN229). Normal brain tissue specimens served as control and were derived from autopsy material archived at the Institute of Neuropathology, University Hospital Zurich (Zurich, Switzerland). According to the Ethics Committee of the Canton Zurich, no ethical approval was necessary, as the tissue samples were anonymized and used for antibody validation and quality control of antibody immunostainings. MCF-7 and HT-29 were purchased from the American Type Culture Collection (Manassas, VA, USA) and the glioma cell lines

LN18 and LN229 were kindly provided by Dr. N. de Tribolet (Lausanne, Switzerland) [26].

For immunohistochemistry (IHC) analysis freshly cut 3 μ m thick sections of the TMA block were mounted on SuperFrost slides (Menzel Gläser, Braunschweig, Germany). Primary antibodies were incubated overnight at 4°C. The LSAB+System-HRP kit (DAKO, Glostrup, Denmark) was used for primary antibodies against PI3K p110 α (1:200), p-Akt (S473) (1:50), p-S6 (S235/236) (1:200) (all Cell Signaling, Danvers, MA, USA), and PI3K p110 β (1:100; Abcam, Cambridge, United Kingdom) according to the manufacturer's protocol. For antibodies against EGFR (1:50), Akt1 (1:400), and S6 (1:100) (all Cell Signaling, Danvers, MA, USA) N-Histofine Simple Stain MAX PO anti-rabbit (Nichirei Biosciences, Tokyo, Japan) was used as a secondary antibody. *PTEN* primary antibody (Cell Signaling, #9559, 1:100) was applied in an automated stainer (Ventana Medical Systems, Tucson, AZ, USA) according to a standard protocol (pretreatment protocol "CC1m"). Bound antibodies were detected by the peroxidase method using diaminobenzidine as chromogen (760-500, Ultraview DAB Detection Kit; Ventana Medical Systems, Tucson, AZ, USA). Finally, the slides were counterstained with hematoxylin prior to dehydration and coverslipping.

IHC labeling of tumor cells in all samples was evaluated semi-quantitatively by an experienced neuropathologist (C.H.) according to the following criteria: no staining (0), slight staining intensity of up to 30% of cells (1), slight to medium staining intensity of 30–50% of cells (2), medium to strong staining intensity of at least 50% of cells (3). Immunostaining scoring was performed in an entirely blinded fashion.

Cases with clinical follow-up were evaluated with respect to association of clinical factors and immunostaining scores to overall survival (OS). OS was defined as date of diagnosis to death of any cause or to the date of the last visit. Univariable analyses were performed by the Kaplan-Meier method, and the log-rank test was used for comparisons of survival in different groups [27]. Cox-regression analysis was applied to analyze the correlation between immunostaining and OS, adjusted for age, gender, and WHO grade (for this analysis immunostaining scores were dichotomized as immunopositive versus immunonegative). Further, the number of positive immunostainings was summarized for both cascades (EGFR, PI3K p110 α / β , Akt1/p-Akt (S473)) and (*PTEN*, Akt1/p-Akt (S473)), with negative values for positive *PTEN* staining since *PTEN* antagonizes the activation of the signaling pathway. This sum score was then compared between S6 and p-S6 (S235/236) positive and negative patients, using permutation tests and bootstrap means based on 10'000 repetitions. All statistical analyses were intended to be rather exploratory than confirmatory and nominal p-values are reported, without adjustment for multiple testing. p-values <0.05, two-tailed are considered statistically significant. Statistical analyses were performed using SAS (Version 9.2 for Windows; SAS Institute Inc., Cary, NC, USA) and PASW Statistics 18 for Windows (SPSS Inc., Chicago, IL, USA).

Cell Lines and *ex vivo* Cultures

GBM cell lines were obtained from the American Type Culture Collection and from Professor M. E. Hegi (CHUV, Lausanne, Switzerland). *PIK3CA* mutated GBM cell lines SK-MG-17 (V344G) and SK-MG-26 (H1074Y) were kindly provided by Professor G. Ritter (Ludwig Institute for Cancer Research, New York Branch at MSKCC, New York, NY, USA) and have been previously described [21]. GBM *ex vivo* cultures were established at the Department of Neurosurgery of the University Hospital Zurich (Zurich, Switzerland) as previously described [28]. All procedures

conducted during the establishment of the *ex vivo* cultures were in accordance with the Declaration of Helsinki and approved by the ethics committee of the Canton Zurich. Informed written consent was obtained from all patients. All GBM cell lines and *ex vivo* cultures were cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich, Buchs, Switzerland) supplemented with 10% (v/v) heat-inactivated fetal calf serum, L-glutamine (Sigma-Aldrich, Buchs, Switzerland), penicillin/streptomycin (GIBCO, Life Technologies Europe, Zug, Switzerland), and 10 μ g/ml gentamycin (Sigma-Aldrich, Buchs, Switzerland).

Western Blotting

Protein expression was analyzed by immunoblotting as described previously [29] using antibodies against EGFR, PI3K p110 β , PI3K p110 δ , Akt1/2/3, caspase 3, ICAD, ERK (all Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), PI3K p110 α , p-Akt (S473), p-Akt (T308), p-S6 (S235/236), p-S6 (S240/244), S6, PARP, p-ERK (all Cell Signaling, Danvers, MA, USA), and β -actin (Sigma-Aldrich, Buchs, Switzerland). Western blot analysis was usually performed in duplicates or triplicates and representative blots are depicted.

Pharmacological Treatments and RNA Interference

Growth factors EGF, PDGF, and IGF-1 were obtained from Calbiochem (Merck (Schweiz) AG, Zug, Switzerland). YM024 [30], PIK75 [31], TGX221 [32], and IC87114 [33] were generously provided by Professor S. P. Jackson (The Australian Centre for Blood Diseases, Monash University, Melbourne, Australia). A66 was previously described [34]. BEZ235 [35] was obtained from ChemieTek (Indianapolis, IN, USA). For growth factor stimulation, cells were pretreated with 0, 1 μ M YM024, or 1 μ M TGX221 for 1 h. GBM cells were transfected with siRNA pools (siGENOME SMART pool siRNA reagents; Dharmacon, Lafayette, CO, USA) targeting PI3K p110 α , PI3K p110 β , PI3K p110 δ , Akt1, Akt2, Akt3, S6K1, or Rac1 using DharmaFECT siRNA transfection reagents (Dharmacon, Lafayette, CO, USA) according to the manufacturer's protocol. Dharmacon's siGENOME Non-Targeting siRNA Pool #2 (SCR) and TOX transfection control (TOX) were used as negative, non-targeting and positive transfection control, respectively.

Cell Proliferation and Anchorage-independent Growth

Cell proliferation was assessed using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, Dübendorf, Switzerland) according to the manufacturer's protocol. Anchorage-independent growth was analyzed using soft agar assay. In short, cells were grown in 0.35% agar supported on a 0.5% agar layer in 6-well plates. Cells were overlaid with medium or the appropriate treatment weekly. After 28 days, colonies were stained with crystal violet and the number of colonies was counted. Cell proliferation and anchorage-independent growth assays were performed in quadruplets and triplicates, respectively. Individual experiments were repeated up to three times, as indicated in the corresponding figure legends.

Flow Cytometry

Apoptotic cells were assessed by flow cytometry as described previously [36]. Adherent and floating cells were collected, stained with FITC-labeled Annexin V (Biotium, Hayward, CA, USA) and propidium iodide (PI), and analyzed on a BD LSR II flow cytometer using BD FACSDiva software (Version 6.1.3; Becton Dickinson AG, Allschwil, Switzerland) and FlowJo software (Version 5.4+; Tree Star, Inc., Ashland, OR, USA). Flow

cytometry analysis measurements were performed in singlet with three repetitions of individual measurements.

Cell Migration

For *in vitro* wound healing assays, a scratch was introduced into a confluent layer of cells using a 200 μ l pipette tip. The medium was replaced with the appropriate treatment medium and plates were further incubated. Photos were taken under a Nikon Eclipse TS100 microscope (4 \times magnification) supplied with a Nikon DXM1200 digital camera (Nikon ACT-1 2.70 software). Open image area was quantified using TScratch software [37] and wound closure was calculated by (open image area before treatment - open image area after treatment). Random migration experiments were performed as described [38]. Cell migration experiments were performed in quadruplets and individual experiments were repeated up to three times, as indicated in the corresponding figure legends.

Chick Chorioallantoic Membrane Assay

Fertilized chicken eggs (*gallus gallus*) from a local hatchery were incubated in a humidified incubator at 37°C. On embryonic day 3 (ED3), a window was opened in the shell and covered again with tape to continue incubation. On ED7, a silicon ring was placed on the chorioallantoic membrane (CAM), and around 3 million T98G cells in 20 μ l PBS were applied after gentle laceration of the membrane. On ED10, photos were taken under a Leica M205 FA microscope (10 \times magnification) supplied with a Canon EOS 5D Mark II camera (Canon EOS Utility software) and tumors were treated with 20 μ l of PI3K p110 α -specific inhibitor PIK75 for four consecutive days. On ED14, post-treatment photos were acquired and the embryos were sacrificed. Tumor volume was estimated by $V = \frac{4}{3}\pi r^3$, with $r = \frac{1}{2}\sqrt{(d1 \times d2)}$ [39].

Statistical Analysis of in vitro and in vivo Experiments

All statistical analyses of the *in vitro* and *in vivo* experiments were performed using Instat+ for Windows (Version 3.036; Statistical Services Centre, University of Reading, Reading, United Kingdom). Statistical significance between multiple groups was tested with ANOVA and $p < 0.05$ was considered significant. Statistical significance between two groups was (subsequently) assessed by two-sided, two-sample Student's *t*-test. For experiments where mean values were calculated over experiments with relative values two-sided, one-sample Student's *t*-tests were performed. The individual statistical tests are indicated in the corresponding figure legends.

Results

Glioma TMA and Correlation Studies

To investigate the expression of different components of the PI3K/Akt signaling pathway in human glioma tissue, we performed IHC analysis on a glioma TMA, containing samples of 103 patients, ranging from WHO grade I (pilocytic astrocytoma) to WHO grade IV (GBM) (Figure 1A and Table S1). Median follow-up time across all patients was 1.6 years (589 days). Complete disease information and IHC staining was available for 74 patients, while the other 29 patient were either lacking survival data or complete IHC staining. Demographics and characteristics of the 74 patients with complete information are summarized in Table S1.

Univariable analysis (Table S2) revealed that patients with high grade tumors (WHO grade $> II$) had a poorer 2-year OS when compared to patients with low grade tumors (WHO grade $\leq II$;

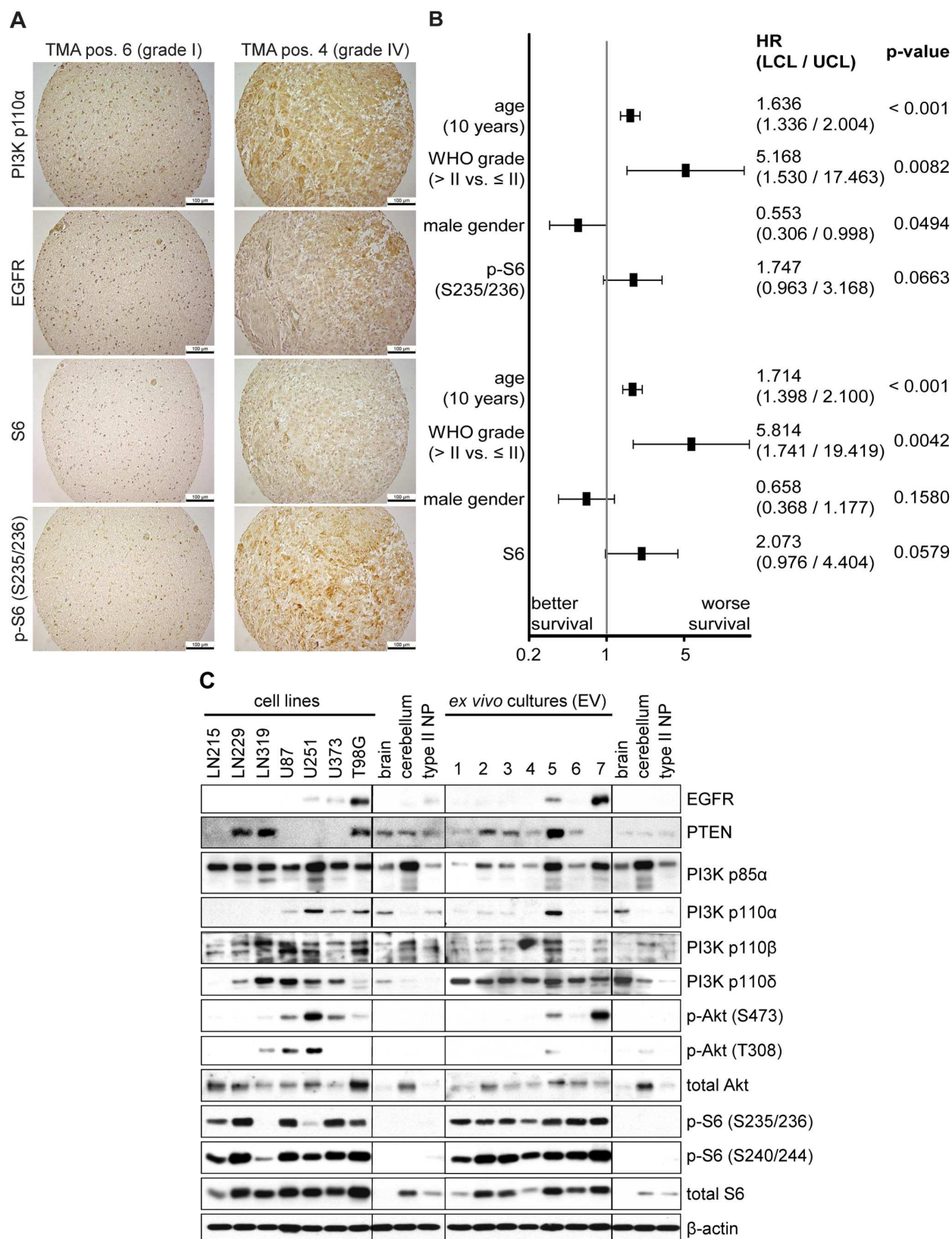


Figure 1. Expression analysis of proteins in the PI3K/Akt/mTOR signaling pathway in glioma and GBM samples. (A) IHC staining of glioma tissue microarray demonstrating increased expression of all antigens shown in GBM (WHO grade IV) compared to pilocytic astrocytoma (WHO grade I). Bars represent 100 μ m. (B) Multivariable Cox-regression of IHC factors in 74 glioma patients by individually adding the immunostaining status to the clinical factors age, gender, and WHO grade. Results for p-S6 (S235/236) and S6 are shown in the upper and lower panel, respectively. HR: hazard ratio, LCL/UCL: lower/upper boundary of 95% confidence interval for HR. (C) Western blot analysis of protein levels of EGFR, PTEN, class I_A

PI3K isoforms, and downstream signaling proteins Akt and S6 in human GBM cell lines and *ex vivo* cultures. Normal human brain and cerebellum tissue as well as non-transformed type II human pneumocytes were used as controls.
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32.2% versus 85.7%, $p < 0.001$), which is consistent with previously described findings and the current state of knowledge [1].

Figure 1A shows examples of IHC staining with PI3K p110 α , EGFR, S6, and p-S6 (S235/236) on two TMA positions, representing stainings of a WHO grade I pilocytic astrocytoma and a WHO grade IV glioblastoma and indicating increased expression of all antigens shown in case of GBM. The majority of glioma samples in the current study were immunopositive for PI3K p110 α (73% of all glioma and 83% of GBM), while detection of PI3K p110 β expression by IHC was infrequent ($n = 1$) and detection of PI3K p110 δ was not possible due to the lack of suitable antibodies.

Immunopositive tumors for EGFR (80%) and p-S6 (S235/236) (42%) were frequently observed. The estimated 2-year OS for patients with p-S6 (S235/236) immunopositive tumors (22.6% versus 57.1%, $p = 0.0043$), with S6 immunopositive tumors (11.1% versus 46.9%, $p = 0.0216$), or with EGFR immunopositive tumors (OS 37.9% versus 60.0%, $p = 0.0451$) was less favorable when compared to patients with immunonegative tumors. Detection of p-Akt (S473) expression by IHC was infrequent ($n = 1$) in this particular cohort, while immunonegative tumors for PTEN were frequently observed (80%).

We next investigated the impact of immunostaining of molecular factors on OS adjusted for important clinical factors such as patients' age, gender, and WHO grade. We added individually the immunostaining status to the clinical factors and applied multivariable Cox-regression analysis to estimate the effect of immunostaining. We found that there was a trend for S6 and p-S6 to be independently associated with worse OS when adjusted for important clinical factors. (Figure 1B; the effects for p-S6 (S235/236) and S6 are shown in the upper and lower part, respectively).

To investigate which cascades (activation through EGFR and/or through PTEN) contribute to the downstream activation/phosphorylation of S6, we summarized the number of immunopositive staining of proteins in both cascades (EGFR, PI3K p110 α/β , Akt1/p-Akt (S473)) and (PTEN, Akt1/p-Akt (S473)), using negative values for positive PTEN staining since PTEN antagonizes the activation of the PI3K/Akt/mTOR pathway. This analysis showed that patients with S6 positive tumors had higher scores in the first cascade (mean difference = 0.77, $p = 0.0086$) as well as in the second cascade (mean difference = 0.477, $p = 0.0346$). Contrary, patients with p-S6 (S235/236) positive tumors had higher score values in the first cascade (mean difference = 0.43, $p = 0.0309$) but not in the second cascade (mean difference = 0.2672, $p = 0.0805$), indicating that downstream signaling was altered in our patient cohort by EGFR/PI3K. The regulatory importance of PTEN was underlined when the scores of the first pathway were compared in patients without PTEN expression (PTEN IHC scoring = 0). In this subgroup of patients those with S6 positive tumors had lower scores in the first cascade (mean difference = 0.707, $p = 0.0905$); similarly p-S6 (S235/236) positive tumors had lower score values in the first cascade (mean difference = 0.48, $p = 0.0660$). Since the number of patients with PTEN expression (PTEN IHC scoring > 0) was too small ($n = 15$), the cascade analysis could not be performed for this subgroup of patients.

Taken together, clinical factors (WHO-grade, gender, and age), and molecular factors (immunopositive tumors for S6 and p-S6 (S235/236)) are associated with OS. Our data provide evidence

that the PI3K/mTOR/S6 signaling pathway can be activated through expression of EGFR/PI3K p110 α rather than through reduced expression of PTEN in glioma.

Expression of PI3K Isoforms in GBM Cell Lines and *ex vivo* Cultures

To gain an overview of the expression pattern of class I_A PI3K isoforms and the activation of the PI3K/Akt/mTOR signaling pathway in GBM cell lines and *ex vivo* cultures, we performed Western blot analysis on a set of seven GBM cell lines and seven *ex vivo* (EV) cultures (Figure 1C and Tables S3 and S4). Protein expression levels in GBM samples were compared to normal human brain and cerebellum tissue as well as to immortalized type II pneumocytes. While expression of the regulatory subunit PI3K p85 α was comparable to levels observed in control lysates, differences in expression of the catalytic subunits PI3K p110 α , PI3K p110 β , and PI3K p110 δ were found. PI3K p110 α showed the most striking variation with some samples showing overexpression (U251, T98G, EV5), while expression of this isoform was nearly absent in certain cell lines (LN215, LN229, LN319) and *ex vivo* cultures (EV1-4, EV6-7). The expression of the PI3K isoform p110 β was largely comparable to levels detected in control samples. Interestingly, PI3K p110 δ was overexpressed in more than half of all GBM cell lines analyzed (LN319, U87, U251, U373), while the expression levels observed in *ex vivo* cultures were comparable to the controls. Basal activation of Akt was detected more frequently in cell lines (LN319, U87, U251, U373, T98G) than in *ex vivo* cultures (EV5, EV7) when compared to control samples. The most striking difference was found upon analysis of S6 protein activation. Strong basal phosphorylation of this protein was observed in all GBM cell lines and *ex vivo* cultures analyzed when compared to control lysates.

Taken together, this expression analysis revealed that individual catalytic class I_A PI3K isoforms are overexpressed in GBM cell lines and *ex vivo* cultures. Moreover, basal activation of the PI3K/Akt/mTOR pathway was detected in the samples analyzed. While phosphorylation of Akt was only observed in a subset of GBM cell lines and *ex vivo* cultures, activation of S6 protein was observed in all samples included in this study. These results confirm that the PI3K/Akt/mTOR signaling pathway is aberrantly activated in GBM.

Inhibition of PI3K Isoform p110 α Impairs GBM Cell Proliferation and Anchorage-Independent Growth

Based on the importance of PI3K/Akt signaling and the deregulation of class I_A PI3K isoforms, especially PI3K p110 α , we investigated the potential of pharmacological inhibition of these isoforms in GBM cell proliferation. GBM cell lines and *ex vivo* cultures were treated with isoform-specific inhibitors YM024, A66, PIK75 (all PI3K p110 α), TGX221 (PI3K p110 β), or IC87114 (PI3K p110 δ) for 72 h (Figure 2A and Figure S1A). Published IC₅₀ values for PI3K p110 inhibition of these inhibitors are as follows: 300 nM for YM024 (PI3K p110 α) [30], 32 nM for A66 (PI3K p110 α) [34], 6–7.8 nM for PIK75 (PI3K p110 α) [31,34], 5–12 nM for TGX221 (PI3K p110 β) [30–32,34], and 41–500 nM for IC87114 (PI3K p110 δ) [30,31,33,34]. While inhibition of PI3K p110 β or PI3K p110 δ did not show any significant effect on cell proliferation of GBM cells, inhibition of PI3K p110 α , especially when using inhibitor PIK75 (the inhibitor with the

lowest *in vitro* IC₅₀ value), led to a dose-dependent decrease in cell proliferation (Figure 2A and Figure S1A). To complement these studies, GBM cells were transiently transfected with siRNA targeting the three class I α PI3K isoforms (*PIK3CA*, *PIK3CB*, or *PIK3CD*). The transfection with siRNAs led to a significant downregulation at the mRNA and protein level (Figure S2). In contrast to GBM cells with transiently downregulated PI3K p110 β or PI3K p110 δ , GBM cells transiently downregulated for PI3K p110 α showed impaired cell proliferation (Figure 2B and Figure S1B). This confirms the results obtained with isoform-specific pharmacological inhibitors and shows that PI3K p110 α contributes to GBM cell proliferation.

Since PI3K p110 α -specific inhibitors have been shown to possess greater activity against tumor cell lines with a *PIK3CA* mutated background [40], we analyzed cell proliferation in the two *PIK3CA* mutated GBM cell lines SK-MG-17 (V344G) and SK-MG-26 (H1047Y) in the presence of either one of the three PI3K p110 α -specific inhibitors YM024, PIK75, or A66 (Figure S3). The two cell lines possess different *PIK3CA* mutations: SK-MG-26 (H1047Y) possesses a rare mutation at a hot spot in the C-terminal portion of the kinase domain (H1047) which is known to lead to a gain of function, while SK-MG-17 (V344G) possesses a rare and not well characterized mutation in the C2 domain which is thought to be part of the membrane-binding region of PI3K p110 α [21,41]. When compared to the *PIK3CA* wild type GBM cell line T98G, SK-MG-26 (H1047Y) displayed a higher sensitivity towards the PI3K p110 α -specific inhibitors, which was, however, not the case for SK-MG-17 (V344G).

Furthermore, anchorage-independent growth of GBM cells was analyzed by means of a soft agar assay in the presence of class I α PI3K isoform-specific inhibitors. Treatment with three different PI3K p110 α -specific inhibitors (YM024, A66, or PIK75) led to a concentration-dependent decrease in the ability of GBM cells to form colonies in soft agar (Figure 2C). Inhibition of PI3K p110 β (TGX221) also decreased the number of colonies formed (Figure 2C), whereas the inhibition of PI3K p110 δ (IC87114; Figure S4A) did not show significant effects on colony formation in soft agar. These observations indicate that the PI3K isoforms p110 α and PI3K p110 β play an important role in anchorage-independent growth of GBM cells, and thus potentially in their tumorigenic potential.

We further investigated cell proliferation and anchorage-independent growth of GBM cells in the presence of the dual PI3K/mTOR inhibitor BEZ235 (Figure 2D). A comparison of these results with the PI3K p110 α -specific inhibitors showed that cell proliferation and colony formation in soft agar can be significantly impaired by only inhibiting the PI3K isoform p110 α , instead of a combined inhibition of PI3K p110 α and mTOR. Thus, concomitant inhibition of PI3K p110 α and mTOR might not be necessary to impair growth in all GBMs.

Apoptosis

To analyze the cause of reduced cell proliferation of GBM cells upon inhibitor treatment, we performed Western blot analysis of apoptosis markers (caspase 3, ICAD, and cleaved PARP; Figure 3A). According to these blots, the PI3K p110 α -specific inhibitor PIK75 induced PARP cleavage and decreased pro-caspase 3 and ICAD-DIFF45 protein levels after 6 h of treatment, indicating thereby that the reduction in cell proliferation is in part due to the induction of apoptosis. The PI3K p110 α -specific inhibitor YM024 also induced PARP cleavage at a concentration of 10 μ M in one of the GBM cell lines tested. Flow cytometry experiments confirmed the induction of apoptosis by the PI3K p110 α -specific inhibitor PIK75, as observed by an increase in

Annexin-V-positive/PI-negative cells after 8 h of treatment (Figure 3B and C). Furthermore, the PI3K p110 α -specific inhibitor YM024 led to an increase in the number of cells in early apoptosis, but to a lesser extent than PIK75. On the contrary, treatment with the PI3K p110 β -specific inhibitor TGX221 did not lead to a significant increase in the number of cells in early apoptosis (Figure 3C).

PI3K Isoforms p110 α and p110 β Play a Role in GBM Cell Migration

In order to investigate the role of the different class I α PI3K isoforms in GBM cell migration, we performed *in vitro* wound healing assays by introducing a scratch into a confluent layer of cells. Pharmacological inhibition of PI3K p110 α significantly impaired the ability of T98G and EV5 cells to migrate into the open wound area (Figure 4A). Inhibition of PI3K p110 β showed even more prominent effects on GBM cell migration, inhibiting wound closure already at lower concentrations (Figure 4A). In contrast, pharmacological inhibition of PI3K p110 δ had no significant effect on wound closure (Figure S4B). These results indicate that PI3K isoforms p110 α and p110 β are involved in the migratory potential of GBM cells.

To complement these studies, we transiently downregulated individual class I α PI3K isoforms in T98G cells and investigated random migration of these cells. Downregulation of the PI3K isoform p110 β (*PIK3CB*) negatively affected cell speed when compared to control cells (SCR), while siRNA-targeting of PI3K p110 α or PI3K p110 δ (*PIK3CA* or *PIK3CD*) had no significant effect (Figure 4B). Further, analysis of the distance covered by T98G cells transfected with siRNA against PI3K p110 β revealed a highly restricted area of movement when compared to mock-transfected cells (Figure 4C). Thus, PI3K p110 β appears to significantly contribute to the motility of GBM cells.

In order to assess the contribution of downstream signaling components of the PI3K pathway to GBM cell migration, T98G cells were transiently transfected with siRNAs targeting the three Akt isoforms (Akt1-3), the ribosomal protein S6 kinase (S6K1), or the small GTP-binding protein Rac1. Silencing Akt1 and S6K1 significantly reduced the migration speed of T98G cells, while silencing Akt2 or Akt3 did not result in comparable effects (Figure 4D). In contrast, silencing Rac1 significantly enhanced the migration speed of T98G cells (Figure 4D). Thus, Akt1 and S6K1 most likely function downstream of class I α PI3K isoforms in the control of cell migration in GBM cells.

PI3K p110 α and PI3K p110 β Inhibition Impairs Activation of the Akt/mTOR Signaling Pathway

To investigate if pharmacological inhibition of PI3K p110 α successfully attenuates basal Akt/mTOR signaling pathway activation, we performed Western blot analysis of GBM cells treated with three different PI3K p110 α -specific inhibitors (YM024, A66, or PIK75). Indeed, all three inhibitors led to a concentration-dependent decrease in the phosphorylation levels of downstream Akt and S6 protein (Figure 5A). Additionally, targeting of PI3K p110 β with the isoform-specific inhibitor TGX221 also led to a concentration-dependent decrease in the phosphorylation levels of Akt and S6 protein (Figure 5B).

Numerous receptor tyrosine kinases (RTKs) are thought to contribute to the malignant properties of gliomas through autocrine signaling loops. Well documented growth factor systems include the EGF system, the PDGF system and the IGF-I system [9,42,43]. We were thus further interested in evaluating the potential of PI3K p110-specific inhibition by YM024 (PI3K

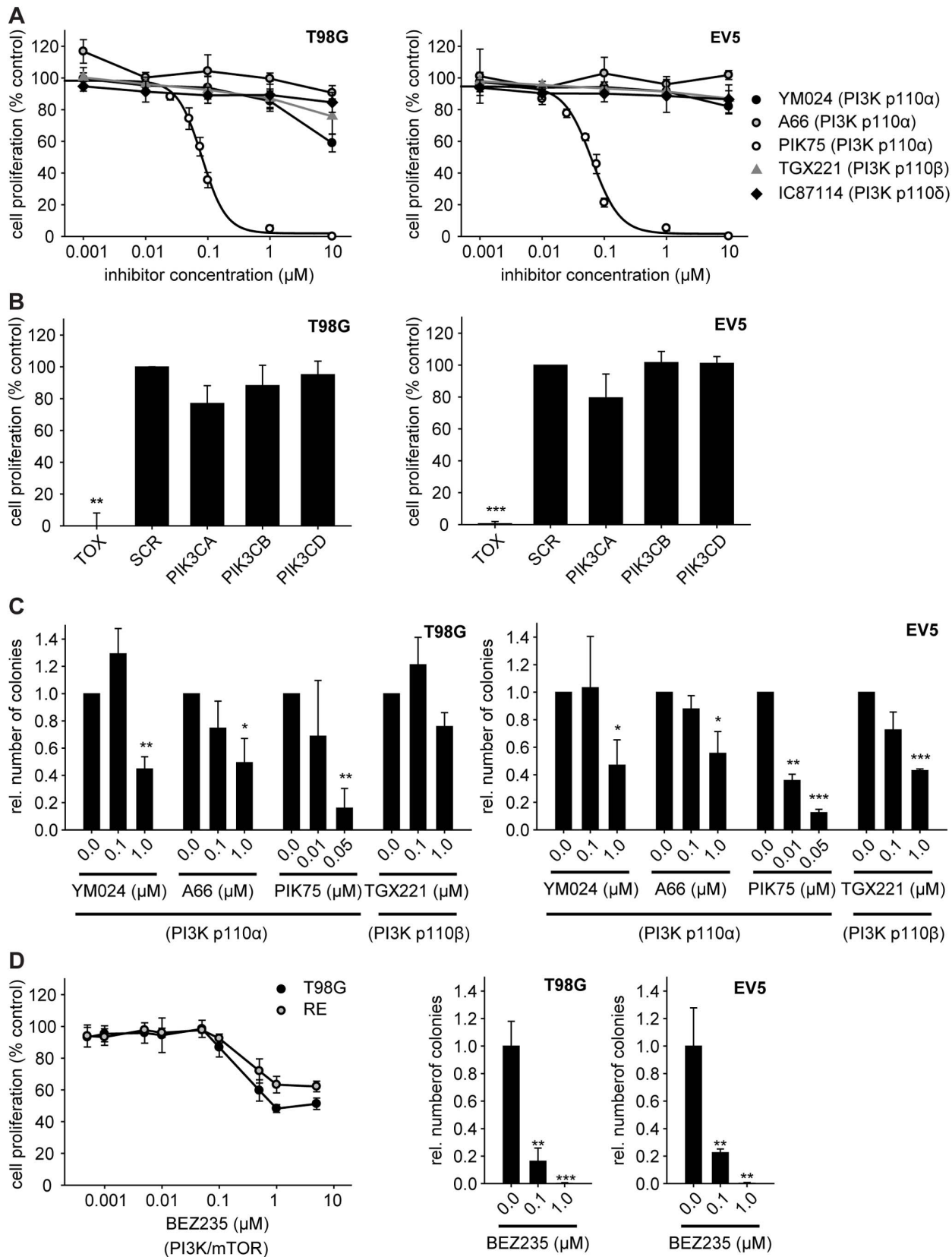


Figure 2. Inhibition of PI3K p110 α impairs GBM cell proliferation and anchorage-independent growth. (A) Cell proliferation of GBM cells in the presence of class I α PI3K isoform-specific inhibitors (72 h). (B) Cell proliferation of GBM cells transiently transfected with siRNA targeting class I α PI3K isoforms p110 α (PIK3CA), p110 β (PIK3CB), or p110 δ (PIK3CD) 48 h post transfection. TOX and SCR siRNAs were used as positive and negative, non-targeting controls, respectively. (C) Anchorage-independent growth (colony formation in soft agar) of GBM cells treated with PI3K p110 α -specific

inhibitors YM024, A66, PIK75, or PI3K p110 β -specific inhibitor TGX221 (28 d). (D) Cell proliferation and anchorage-independent growth of GBM cells in the presence of the dual PI3K/mTOR inhibitor BEZ235 (72 h and 28 d, respectively). Curves and bars represent the means of three individual experiments \pm standard deviation; single experiment for soft agar assay with BEZ235; *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$ compared to 0.0 μ M inhibitor or SCR non-targeting siRNA control as determined by two-sided, one-sample Student's *t*-tests.
doi:10.1371/journal.pone.0094132.g002

p110 α) or TGX221 (PI3K p110 β) to affect growth factor-induced pathway activation. Indeed, pre-treatment of T98G and EV7 cells with YM024 impaired phosphorylation of Akt and S6 protein in response to EGF, PDGF, or IGF-1 stimulation (Figure 5C). Contrary to pharmacological inhibition of PI3K p110 α , treatments with the PI3K p110 β -specific inhibitor TGX221 only partially decreased phosphorylation of the ribosomal S6 protein and not to the same extent as the PI3K p110 α inhibitor YM024. These results implicate that PI3K p110 α plays a crucial role in transmitting signals from activated RTKs and thus could be involved in autocrine/paracrine signaling events in GBM.

Given the known existing cross-talks between the PI3K/Akt/mTOR and the Ras/extracellular signal-regulated kinase (ERK) signaling pathway, we performed Western blot analysis of the GBM cells EV5, T98G, U87 and U251 after treatment with PI3K isoform-specific inhibitors (6 h) and analyzed the phosphorylation levels of ERK. These experiments showed no induction of ERK phosphorylation upon treatment of GBM cells with PI3K isoform-specific inhibitors and thereby indicate no feedback compensation through the Ras/ERK signaling pathway (data not shown).

Pharmacological Inhibition of PI3K p110 α Impairs T98G Tumor Growth *in vivo*

To investigate the potential of PI3K p110 α -specific inhibition on tumor growth, we performed an *in vivo* CAM assay using T98G GBM cells. Deposition of these cells on the CAM resulted in the formation of solid tumors within three days (Figure 6A, before treatment). Since the PI3K p110 α -specific inhibitor PIK75 showed the most potent effects in the *in vitro* experiments, we used this inhibitor in the *in vivo* setting. Indeed, treating the T98G tumors formed on the CAM for four consecutive days with PIK75 led to decreased tumor sizes when compared to control treatment (Figure 6A and B).

Discussion

The development of novel targeted therapies for GBM is of crucial importance in view of the dismal prognosis of patients with current treatment protocols. It has been recognized that the PI3K/Akt/mTOR pathway is a very promising target for the development of novel anti-cancer therapies in human cancer, including GBM [44,45]. The most prominent mutations targeting the pathway in GBM are in *PTEN*, *PIK3CA*, and *EGFR* [11–13,18]. To date, the only agents that have been evaluated in GBM clinical trials are allosteric mTOR inhibitors (analogs of rapamycin, rapalogs), which have not shown significant activity, either as single agents or in combination with standard chemotherapy [11,44]. This may be explained by various factors, including the multiple molecular mechanisms that exist which enable tumors to develop resistance to allosteric mTOR inhibitors [46]. Therefore, agents targeting Akt, PI3K, and mTOR may represent more promising drugs to inhibit this pathway in GBM patients [46,47]. Indeed, there exist several reports on the pre-clinical activity of Akt, PI3K, and mTOR inhibitors in GBM models, and several clinical trials have been initiated with these novel agents [11,44,45]. Concerning PI3K targeting, it has been generally accepted that the dual targeting of PI3K p110 α and mTOR is the most potent strategy to inhibit the pathway in a variety of human

cancers, including GBM, by circumventing some of the feedback loops which control tumor resistance to mTOR inhibitors [48]. In addition, targeting class I PI3K isoforms has been recently shown to have activity in GBM models, which was dependent on *TP53* status [49]. Targeting the class I α PI3K isoform p110 δ was also recently shown to impair the migration of GBM cells [50]. Another set of reports has demonstrated that PI3K p110 β may be the most relevant PI3K isoform to target in cancers which have mutations in *PTEN*, such as GBM and prostate cancer [51,52]. So far there is no consensus on the most relevant predictive biomarkers for PI3K inhibitors in GBM, although there are reports demonstrating that cancers with *PIK3CA* mutations are most sensitive to these agents [53,54].

In this report, we show by IHC staining of a glioma TMA that PI3K p110 α is the class I α PI3K isoform which displays the most consistent expression in GBM patient samples, in contrast to PI3K p110 β . Due to the lack of suitable antibodies, it was not possible to assess the expression of PI3K p110 δ , which was previously reported to be overexpressed at the mRNA level in primary GBM [13,22]. In cell lines and *ex vivo* cultures, all three class I α PI3K isoforms were expressed, indicating that GBM cell lines and *ex vivo* cultures may up-regulate the expression of some PI3K isoforms, such as PI3K p110 β . When evaluating the contribution of the expression of components of the EGFR/PI3K/Akt/mTOR pathway to patient survival, it was found that phosphorylation of ribosomal S6 protein was the best predictor of reduced patient survival. In addition, co-expression of the EGFR/PI3K p110 α /p-S6 module was related to patient survival, indicating that the activation of the pathway can influence GBM outcome. Interestingly, this phenomenon was independent of *PTEN* and p-Akt status, indicating that there exists a group of GBM tumors in which the EGFR/PI3K/mTOR pathway is active by increased expression of some components, such as EGFR and PI3K p110 α . On the other hand, GBM characterized by reduced or absent *PTEN* expression may rely on another PI3K isoform, such as PI3K p110 β [51,52]. There are several reports which have investigated the prognostic value of mutations in genes involved in the PI3K signaling pathway as well as the activation status of Akt and the ribosomal S6 protein in GBM. While there is no consensus on the prognostic value of *PTEN* mutations or expression, the phosphorylation status of the ribosomal S6 protein has been previously inversely correlated to patient survival [7,55–57].

In view of the consistent expression of PI3K p110 α in primary GBM samples and its association with EGFR and mTOR pathway activation, we next evaluated a panel of pharmacological isoform-specific inhibitors in GBM cell lines and *ex vivo* cultures expressing the EGFR/PI3K p110 α /p-S6 module. In comparison to the dual PI3K p110 α /mTOR inhibitor BEZ235, PI3K p110 α -specific inhibitors displayed less potent activity when evaluated on cells growing under adherent and optimal serum conditions. The activity of the three PI3K p110 α inhibitors in cells reflected their potency for inhibition of PI3K p110 α kinase activity *in vitro*. In addition, some of the *PIK3CA* mutant GBM cell lines SK-MG-26 (H1047Y) responded better to the PI3K p110 α -specific inhibitors than GBM cell lines where *PIK3CA* is wild type. *PIK3CA* mutations at position H1047 have been reported to predict sensitivity to inhibitors of the PI3K pathway [53], which may explain the higher sensitivity of the SK-MG-26 (H1047Y) cell line towards PI3K

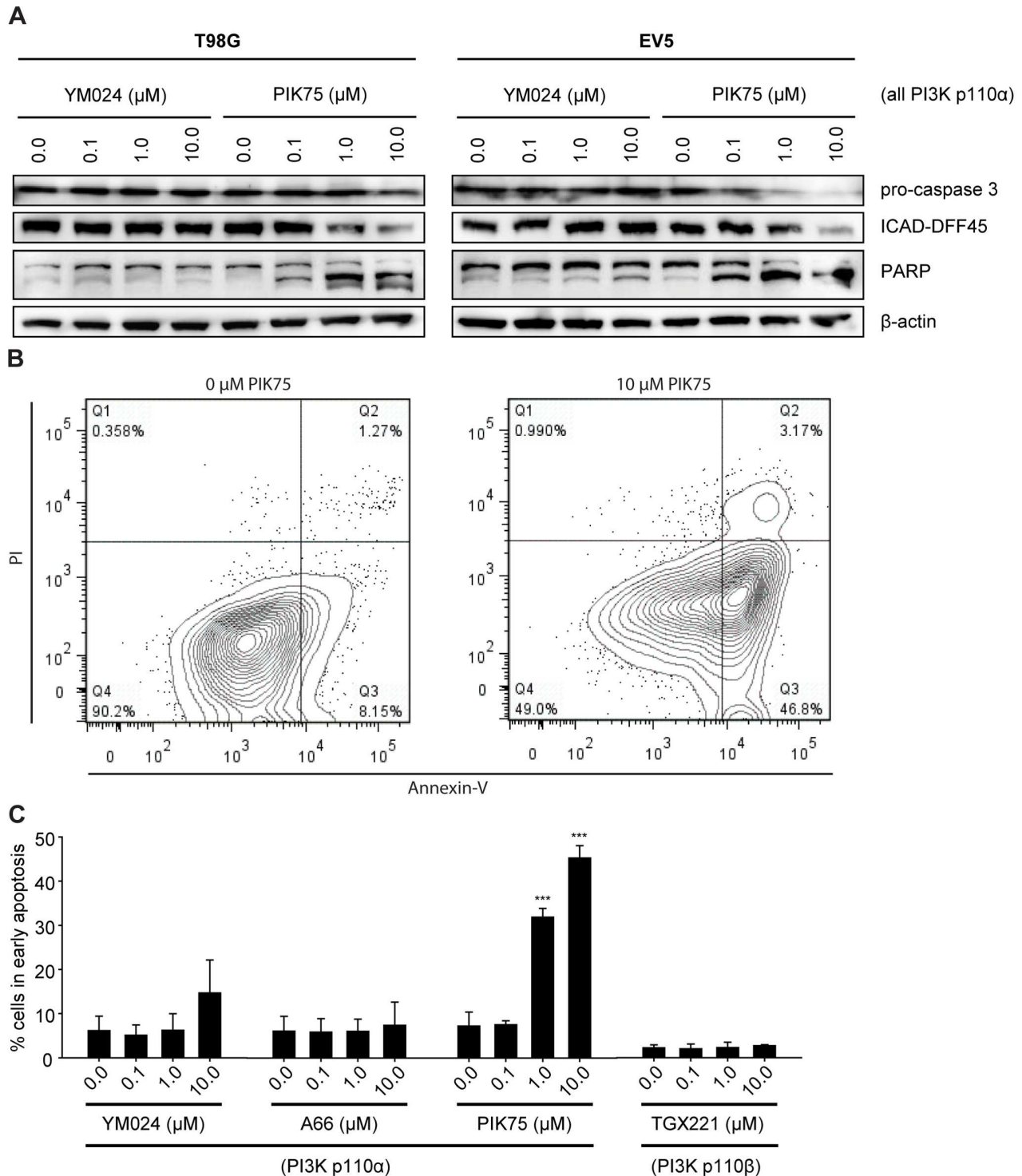


Figure 3. Inhibition of the PI3K isoform p110 α with isoform-specific inhibitor PIK75 induces apoptosis. (A) Western blot analysis of apoptosis markers of GBM cells following treatment with increasing concentrations of PI3K p110 α -specific inhibitors YM024 or PIK75 (6 h). (B) Flow cytometry analysis of T98G cells after treatment with PI3K p110 α -specific inhibitor PIK75 (8 h) stained with Annexin-V and PI. (C) Quantification of Annexin-V-positive/PI-negative T98G cells after treatment with PI3K p110 α -specific inhibitors YM024, A66, PIK75, or PI3K p110 β -specific inhibitor TGX221 (8 h). Bars represent the means of three or two individual experiments \pm standard deviation for PI3K p110 α - or PI3K p110 β -specific inhibitors, respectively; ***, $p \leq 0.001$ compared to 0.0 μ M inhibitor as determined by two-sided, one-sample Student's t -tests. doi:10.1371/journal.pone.0094132.g003

p110 α -specific inhibitors. However, the activity of the PI3K p110 α inhibitors was not influenced by PTEN status. This was confirmed by the observation that RNAi targeting PI3K p110 α partially

reduced cell proliferation under adherent and optimal serum conditions. In contrast, when assayed under anchorage-independent conditions, the PI3K p110 α and PI3K p110 β inhibitors had

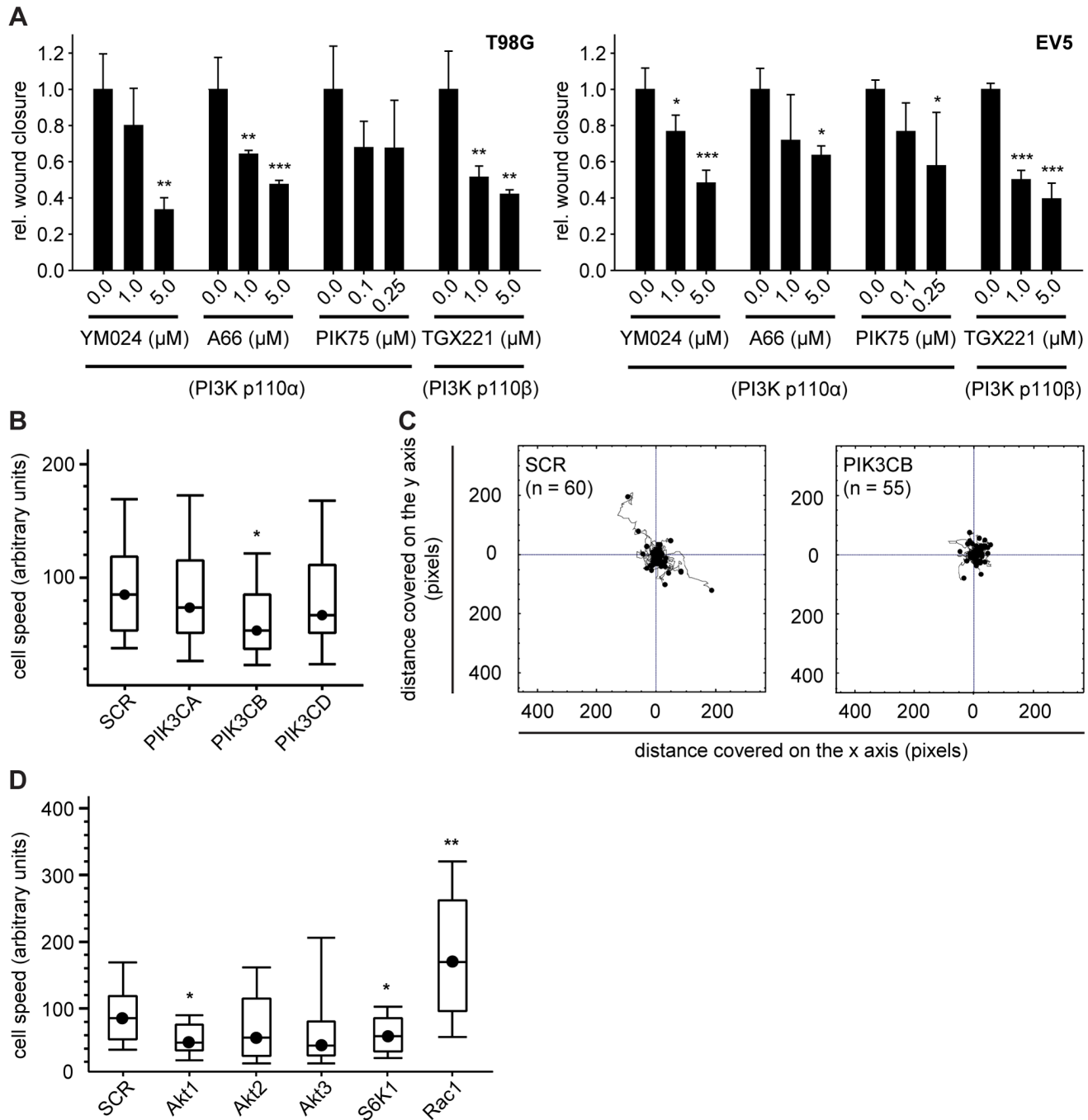


Figure 4. Class I_A PI3K isoforms p110 α and p110 β play a role in GBM cell migration. (A) Analysis of the migratory potential of GBM cells by means of wound healing assays in the presence of PI3K p110 α or PI3K p110 β -specific inhibitors (18 h). Bars represent the means of three individual experiments \pm standard deviation; *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$ compared to 0.0 μ M inhibitor as determined by two-sided, one-sample Student's *t*-tests. (B) Migration speed of T98G cells in a random migration experiment following downregulation of class I_A PI3K isoforms by siRNA. *: $p \leq 0.05$ compared to SCR non-targeting siRNA as determined by two-sided, one-sample Student's *t*-test. (C) Distance of migration in a random migration experiment of T98G cells transfected with non-targeting control (SCR) or PI3K p110 β -targeting siRNA (PIK3CB). (D) Migration speed of T98G cells in a random migration experiment following downregulation of PI3K/Akt signaling pathway molecules by siRNA. *: $p \leq 0.05$, **: $p \leq 0.01$ compared to SCR non-targeting siRNA as determined by two-sided, two-sample Student's *t*-tests. doi:10.1371/journal.pone.0094132.g004

single agent activity in different GBM cell lines and *ex vivo* cultures. In contrast, PI3K p110 δ inhibitors or siRNA did not show a similar broad response in GBM, either under adherent or anchorage-independent growth conditions. Furthermore, the observation that PI3K signaling pathway inhibition impairs

anchorage-independent growth of GBM cells, by targeting PI3K p110 α or PI3K p110 β , complements previously published data investigating the role of Akt3 with respect to anchorage-independent growth in GBM cells [58,59]. These investigations show that GBM cell lines are dependent on Akt3 by means of

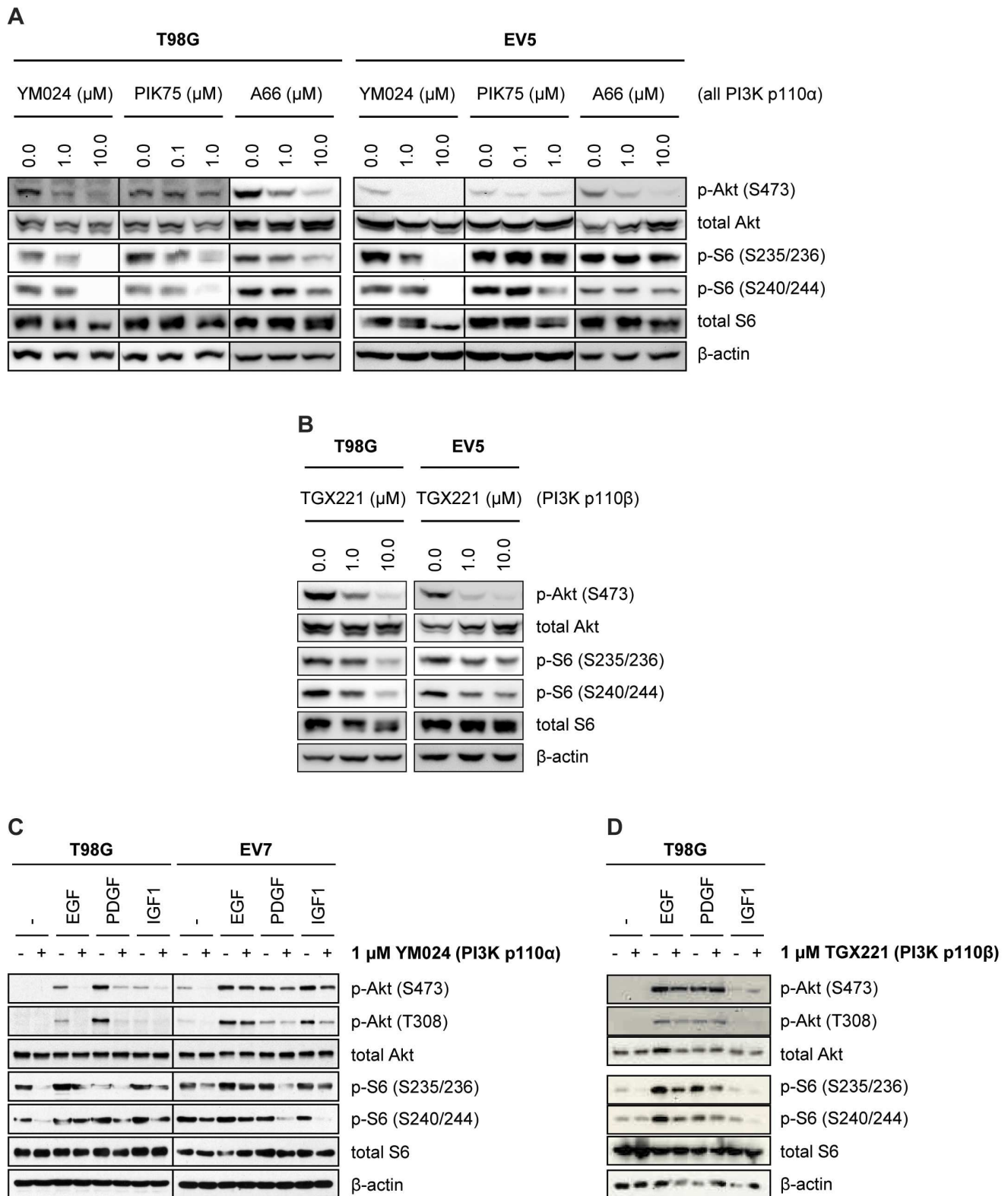


Figure 5. Pharmacological inhibition of PI3K p110 α and PI3K p110 β impairs downstream signaling. (A) Western blot analysis of basal Akt/mTOR signaling activation by detection of phosphorylated downstream proteins Akt and S6 in GBM cells following treatment with increasing concentrations of PI3K p110 α -specific inhibitors YM024, PIK75, or A66 (6 h). (B) Western blot analysis of phosphorylated downstream proteins Akt and S6 protein of GBM cells following treatment with increasing concentrations of the PI3K p110 β -specific inhibitor TGX221 (6 h). (C) Growth factor-induced PI3K/Akt signaling activation after pretreatment of T98G and EV7 cells with PI3K p110 α -specific inhibitor YM024. (D) Growth factor-induced PI3K/Akt signaling activation after pretreatment of T98G cells with PI3K p110 β -specific inhibitor TGX221.

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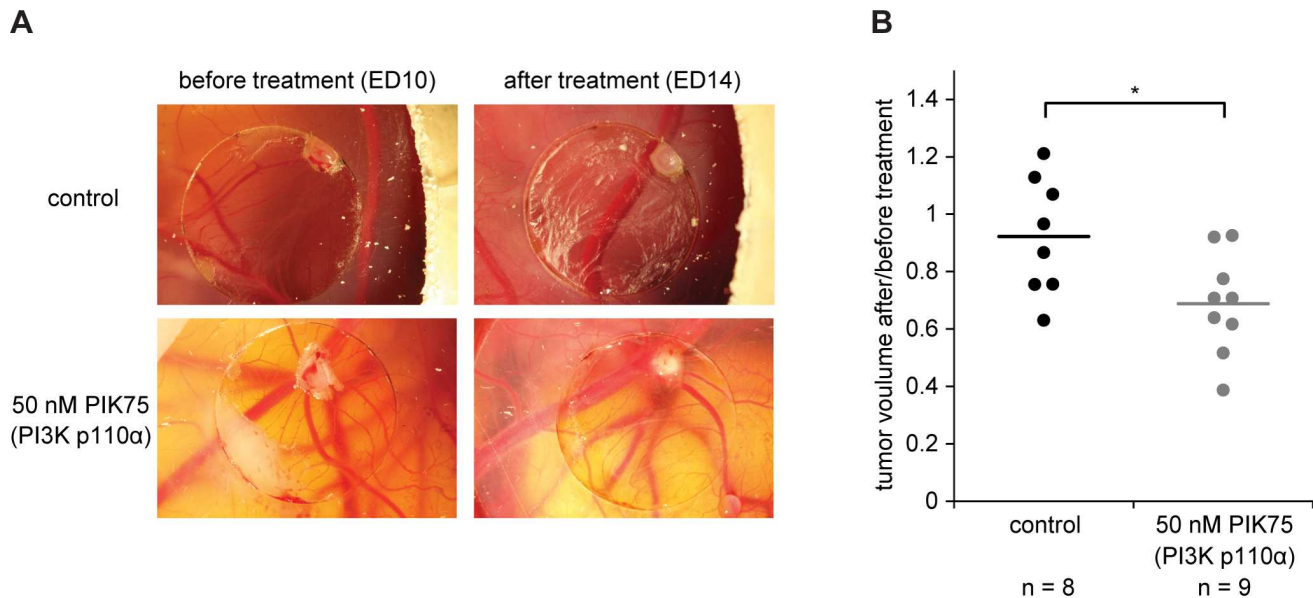


Figure 6. PI3K p110 α -specific inhibition of T98G tumors formed on the chick chorioallantoic membrane impairs tumor growth. (A) Representative pictures of T98G tumors formed on the CAM three days post cell application (10 \times magnification). Tumors were treated with PIK75 as indicated for four consecutive days. (B) Quantification of changes in tumor volume before and after PIK75 or control treatment. Lines indicate the mean of each group. *: $p = 0.02$ compared to control treatment as determined by two-sided, two-sample Student's t -test. doi:10.1371/journal.pone.0094132.g006

siRNA or shRNA downregulation of Akt3 and analysis of anchorage-independent growth in soft agar.

When investigating the role of PI3K isoforms in GBM cell migration, PI3K p110 β appeared to play a major role. In contrast to a recent report [50], PI3K p110 δ targeting by siRNA or pharmacological inhibitors did not significantly impair GBM cell migration. Contrary to previously published data stating that cell migration of T98G GBM cells is dependent on Akt3 rather than Akt1 or Akt2 [58], our assay showed that T98G GBM cell migration depends on Akt1 rather than Akt2 or Akt3. The discrepancy to this publication could be explained by the different experimental setups: while we were using a random migration assay on plastic with equivalent distribution of nutrients, Endersby *et al.* used a nutrient-driven migration assay through matrigel. Different nutrient environment as well as the presence of matrigel might lead to the expression of different protein levels in the Akt isoforms and might thus result in different cellular responses.

The induction of apoptosis was observed in GBM at high concentrations of the PI3K p110 α inhibitors, indicating that single agent targeting of this isoform is not sufficient to induce cell death under adherent conditions. This observation is supported by previous studies, which have demonstrated that the induction of cell cycle arrest is the most prominent mechanism of action of these drugs, when used as single agents under adherent conditions [35]. When evaluated in an *in vivo* model, the PI3K p110 α inhibitor PIK75 induced tumor regression of GBM, supporting the view that single agent targeting of PI3K p110 α may have anti-tumor effects.

Together our data demonstrate that the PI3K p110 α and PI3K p110 β isoforms appear to differentially contribute to critical cell responses in GBM, which is important when considering the future use of isoform-specific PI3K inhibitors. Drugs targeting PI3K p110 α may be considered for further evaluation in GBM, in particular in tumors which express a functional EGFR/PI3K p110 α /p-S6 signaling module. These findings are important, since at present it is unclear which drugs targeting the PI3K/Akt/

mTOR pathway have the most favorable profile in glioblastoma patients and there are no robust predictive biomarkers for these agents.

Supporting Information

Figure S1 Cell proliferation of GBM cells after targeting of class I α PI3K isoforms. (A) Cell proliferation of GBM cells in the presence of class I α PI3K isoform-specific inhibitors (72 h). (B) Cell proliferation of GBM cells transiently transfected with siRNA targeting class I α PI3K isoforms p110 α (PIK3CA), p110 β (PIK3CB), or p110 δ (PIK3CD). TOX and SCR siRNAs were used as positive and negative, non-targeting controls, respectively. Curves and bars represent the means of three individual experiments \pm standard deviation; *: $p \leq 0.05$, **: $p \leq 0.01$ compared to SCR non-targeting control siRNA as determined by two-sided, one-sample Student's t -tests. (TIF)

Figure S2 Transient transfection of GBM cells with siRNA targeting specific class I α PI3K isoforms. Transient transfection of T98G (A) and EV5 (B) cells with siRNA targeting class I α PI3K isoforms p110 α (PIK3CA), p110 β (PIK3CB), or p110 δ (PIK3CD) leads to target downregulation at the mRNA level (upper panels) and protein level (lower panels) 48 h post transfection. Bars represent the means of three individual experiments \pm standard deviation; **: $p \leq 0.01$, ***: $p \leq 0.001$ compared to SCR non-targeting control siRNA as determined by two-sided, one-sample Student's t -tests (TIF)

Figure S3 PI3K p110 α -specific inhibitor treatment of PIK3CA wild type and PIK3CA mutated GBM cell lines. PIK3CA wild type GBM cell line T98G and PIK3CA mutated GBM cell lines SK-MG-17 (V344G) and SK-MG-26 (H1047Y) were treated with PI3K p110 α -specific inhibitors YM024, PIK75, or A66 (72 h). Curves represent the means of three independent

experiments \pm standard deviation. *: $p \leq 0.05$, **: $p \leq 0.01$ compared to T98G cells as determined by two-sided, one-sample Student's t -tests.

(TIF)

Figure S4 Soft agar colony formation assay and wound healing assay of GBM cells. (A) Anchorage-independent growth (colony formation in soft agar of GBM cells in the presence of PI3K p110 δ -specific inhibitor IC87114 (28 d). (B) Analysis of the migratory potential of GBM cells by means of wound healing assays in the presence of PI3K p110 δ -specific inhibitor IC87114 (18 h). Bars represent the means of three individual experiments \pm standard deviation.

(TIF)

Table S1 Demographics and disease characteristics of 74 patients with complete disease and IHC information.

(PDF)

Table S2 Univariable analyses of 74 patients with complete disease and IHC information.

(DOC)

Table S3 Genetic background information about the GBM cell lines used in this study.

(PDF)

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Table S4 Additional information about the GBM *ex vivo* cultures used in this study.

(PDF)

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Author Contributions

Conceived and designed the experiments: AA KF VD AOB OP KH DB BSR. Performed the experiments: KH DB SD OP PC FS ZL BSR. Analyzed the data: KH DB OP PC ZL FS AT CH BSR AOB. Contributed reagents/materials/analysis tools: PRS VD. Wrote the paper: KH DB AOB AA KF OP AT CH.

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